Defence Research and Development Canada
Recherche et développement pour la défense Canada

DEFENCE R&D DÉFENSE

Expression of *Bacillus anthracis* protective antigen in *Bacillus megaterium*

B.J. Berger, K.E. Schwandt and C.L. Radford
Defence R&D Canada – Suffield

Technical Memorandum
DRDC Suffield TM 2004-045
March 2004

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

Canada

Expression of *Bacillus anthracis* protective antigen in *Bacillus megaterium*

B. J. Berger, K. E. Schwandt, and C. L. Radford
Defence R&D Canada – Suffield

Defence R&D Canada – Suffield

Technical Memorandum

DRDC Suffield TM 2004-045

March 2004

AQ F07-12-12239

20071002087

Author

Dr. B. J. Berger

Approved by

Dr. J. Lavigne

Head, Chemical & Biological Defence Section

Approved for release by

Dr. R. Bide

Chair, DRDC Suffield Document Review Panel

© Her Majesty the Queen as represented by the Minister of National Defence, 2004

© Sa majesté la reine, représentée par le ministre de la Défense nationale, 2004

Abstract

Bacillus anthracis protective antigen is the central component of the anthrax toxin complexes that facilitates entry of lethal factor and edema factor into host cells. Protective antigen is also the major immunogenic component present in the currently licensed anthrax vaccine. In order to produce full-length, soluble protective antigen, the gene has been cloned and expressed using *Bacillus megaterium* and a xylose-inducible heterologous expression system. After only 3.5 hours growth post-induction in Luria-Bertani broth, the transgenic *B. megaterium* were found to secrete approximately 1 µg/ml protective antigen into the culture medium. The recombinant protein was easily purified to homogeneity in a single step by ion exchange chromatography. N-terminal amino acid sequencing of the final product confirmed that the recombinant protective antigen was full-length and that no proteolytic degradation had occurred.

Résumé

L'antigène protecteur *Bacillus anthracis* est le composant central des complexes des toxines du charbon qui facilite l'entrée du facteur létal et d'œdème à l'intérieur des cellules hôtes. Cet antigène protecteur est aussi le composant immunogène essentiel qui est présent dans le vaccin actuellement autorisé contre le charbon. Dans le but de produire un antigène protecteur complet et soluble, le gène a été cloné et exprimé en utilisant le *Bacillus megaterium* et un système d'expression hétérologue inductible au xylose. Après seulement 3 heures et demi de croissance post-induction dans le bouillon Luria-Bertani, on a trouvé que les *B. megaterium* transgéniques sécrètent à peu près 1 µg/ml d'antigène protecteur dans le milieu de culture. La protéine recombinante a été facilement purifiée à l'état d'homogénéité, en une seule étape, par une chromatographie d'échange d'ions. Le séquençage aminoacide N-terminal du produit final a confirmé que l'antigène protecteur recombinant était complet et qu'aucune dégradation protéolytique ne s'était produite.

This page intentionally left blank.

Executive summary

Background

Protective antigen (PA) is an 83 kDa protein excreted by *Bacillus anthracis* that is an essential component of anthrax toxins, and functions by facilitating the entry of lethal toxin and/or edema toxin into host cells. Host antibodies directed against PA are able to prevent the anthrax toxins from entering the target cells and, thus, prevent cell death and human toxicity. For this reason, PA is the primary antigen present in the currently licensed anthrax vaccine, which consists of PA absorbed from *B. anthracis* culture supernatants. It is believed that other anthrax proteins that are also co-purified by this process are responsible for the majority of clinical side-effects seen with the existing vaccine. For this reason, there has been a great interest in the production of a recombinant protective antigen (rPA) vaccine that contains no other anthrax proteins. Pure and easily produced rPA would also be useful as a clinical tool for titering patients who have received the anthrax vaccine in order to determine a more rational time-course for booster administration. Finally, rPA would assist in the development and quality control of hand held test kits, many of which utilise immobilised antibodies against PA. Historically, the production of rPA has been hampered by many of the traditional issues that hinder heterologous expression of proteins: insoluble, inactive inclusion body formation; low protein yields; endotoxin production by the expression host; incompatibility of codon biases; and proteolytic degradation during protein production. The most advanced rPA-based vaccine products are the UK *Escherichia coli* system which must deal with the removal of *E. coli* endotoxin, and the US *B. anthracis* system which utilises a potential pathogen as the expression host.

Results

We have expressed full-length, soluble, active rPA using a *Bacillus megaterium* expression system modified from a commercially available kit. The use of *B. megaterium* solves all of the inherent problems with alternative rPA expression systems: *B. megaterium* is a safe, non-pathogenic, and industry-proven expression host; there are no extracellular proteases to degrade the rPA; *B. megaterium* does not produce endotoxin; *B. megaterium* can be grown easily in a defined, meat-free medium; and the rPA is easily purified to homogeneity. In this system, we have readily produced 1 µg/ml rPA into the culture supernatant over only 3.5 hr of cell growth post-induction, and this material was purified in a single step by ion-exchange chromatography. N-terminal amino acid sequencing definitively proved that the rPA was pure, full-length, and not subjected to proteolytic degradation.

Significance

The use of *B. megaterium* as an expression host for rPA should provide a simpler industrial process for vaccine production, and could lead to a safer anthrax vaccine product. Further studies are recommended in order to increase the yield of rPA in this system.

Berger, B. J., Schwandt, K. E., and Radford, C. L. (2004). Expression of *Bacillus anthracis* protective antigen in *Bacillus megaterium*. (DRDC Suffield TM 2004-045). Defence R&D Canada – Suffield.

Sommaire

Contexte

Un antigène protecteur (AP) est une protéine de 83 kDa excrétée par le *Bacillus anthracis* qui est un composant essentiel des toxines du charbon et fonctionne en facilitant l'entrée de la toxine létale et/ou de la toxine de l'œdème à l'intérieur des cellules hôtes. Les anticorps hôtes dirigés contre les AP sont capables d'empêcher les toxines du charbon d'entrer à l'intérieur des cellules cibles et par conséquent d'empêcher la mort de la cellule et l'effet toxique sur les humains. C'est pour cette raison que l'AP est l'antigène primaire présent dans le vaccin actuellement autorisé contre le charbon et consiste en un AP absorbé à partir des surnageants cultivés du *B. anthracis*. On pense que d'autres protéines de charbon, purifiées par ce même procédé, causent la majorité des effets secondaires ayant été observés avec le vaccin existant. Pour cette raison, on s'est beaucoup intéressé à la production d'un vaccin d'antigène protecteur recombinant (APr) qui ne contient pas d'autres protéines de charbon. Un APr qui est pur et qui peut être produit facilement serait aussi utile comme outil clinique pour faire le titrage des patients qui ont reçu le vaccin contre le charbon et déterminer un temps d'absorption plus rationnel pour savoir quand administrer l'injection du rappel. Enfin, l'APr faciliterait la mise au point et le contrôle de qualité des trousses de tests manuelles, dont plusieurs utilisent des anticorps immobilisés contre l'AP. Historiquement, la production d'APr a été ralentie par beaucoup de problèmes traditionnels entravant l'expression hétérologue des protéines : la formation de corps d'inclusion insolubles et inactifs, la faible production de protéines, la production d'endotoxine par l'hôte de l'expression ; l'incompatibilité de biais de codons ; la dégradation protéolytique durant la production de protéine. Les derniers vaccins à base d'APr sont le système *Escherichia coli* de la GB qui doit effectuer le retrait de l'endoxine *E. coli* et le système *B. anthracis* américain qui utilise un pathogène potentiel comme hôte de l'expression.

Les résultats

On a exprimé un APr complet et soluble et actif en utilisant le système de l'expression *B. anthracis*, ayant été modifiée à partir d'une trousse disponible dans le commerce. L'utilisation du *B. megaterium* est la solution à tous les problèmes inhérents aux systèmes d'expression APr alternatifs : le *B. megaterium* est un hôte d'expression sans danger, non pathogénique et reconnu par l'industrie ; il n'y a pas de protéases extracellulaires qui risquent de dégrader l'APr ; le *B. megaterium* ne produit pas d'endoxine ; il peut se développer facilement dans un milieu défini et ne contient pas de matières animales ; l'APr est ainsi facilement purifié jusqu'à son homogénéité. Dans ce système, on a déjà facilement produit 1µg/ml de systèmes APr dans un surnageant cultivé, et ceci en seulement 3 heures et demi de croissance post-induction des cellules. Ce matériau a aussi été purifié en une seule étape par une chromatographie d'échange d'ions. Le séquençage des aminoacides N-terminal a définitivement prouvé que l'APr était pur, complet et non sujet à la dégradation protéolytique.

La portée de ces résultats

L'utilisation du *B. megaterium* comme hôte d'expression de l'Apr devrait permettre de mettre au point un procédé industriel de production d'un vaccin plus simple et pourrait aboutir à des vaccins plus sécuritaires. On recommande d'approfondir la recherche visant à augmenter la production d'APr dans ce système.

Berger, B. J., Schwandt, K. E., and Radford, C. L. (2004). Expression of *Bacillus anthracis* protective antigen in *Bacillus megaterium*. (DRDC Suffield TM 2004-045). R & D pour la défense Canada – Suffield.

This page intentionally left blank.

Table of contents

List of figures

Acknowledgements

The authors would like to thank Dr. Les Nagata and Dan Fornika for titering the rPA by ELISA. Kerrie Schwandt was a 2003 Defence Research Associate.

This page left intentionally blank.

Introduction

The gram-positive, spore-forming bacterium *Bacillus anthracis* is the causative agent of anthrax. While normally a disease of herbivores, anthrax can also infect humans via the cutaneous, gastrointestinal, or inhalational routes. The latter route of infection is uniformly lethal without therapeutic intervention, and, together with the durability and longevity of *B. anthracis* spores, is the basis for the development of anthrax as a biological warfare agent. The recent distribution of anthrax-laced letters in the United States, and subsequent deaths of several infected individuals, has highlighted the ease of use of anthrax as a bioterror agent and the importance of medical countermeasures [1,2].

Full virulence of *B. anthracis* is dependent on the expression of two major factors encoded by plasmids. The production of a poly-D-glutamic acid capsule, encoded by pXO2, is essential for immune evasion and cellular survival in the host [3,4]. The lethal effects of anthrax are due to the production of two bipartite toxins encoded by pXO1 [5]. The lethal factor (LF) is a Zn^{2+}-protease that is specific for the cleavage of mitogen-activated protein kinase kinases within macrophages, which leads to macrophage death, an associated cytokine cascade, and death of the host in a toxic-shock manner [6]. The edema factor (EF) is a calmodulin-dependent adenylate cyclase that leads to tissue edema and an impairment of neutrophil function [7]. Both toxin proteins are reliant on a third anthrax protein, protective antigen (PA), for entry into the host cell [8]. PA binds to the host cell surface, is cleaved by a furin-related protease to form PA_{63}, which self-associates into heptamers and thus forms a high-affinity binding site for LF or EF. The resulting toxin-PA complex is then endocytosed and the heptameric PA_{63} inserts into the lysosomal membrane, facilitating translocation of the toxin molecule into the cytosol.

It has been long known that cultures of *B. anthracis* excrete PA into the medium during growth, and that this extracellular PA is non-toxic and can induce a protective immune response [9,10]. All currently licensed anthrax vaccines rely on PA as the antigenic component. However, the current method of isolating PA from *B. anthracis* culture supernatants has several disadvantages. Although the strains of anthrax used for vaccine production are non-encapsulated (Sterne or V770-NP1-R), they must be treated as Biohazard Level-2 or Biohazard Level-3 pathogens (depending on the country) and require special production facilities. In addition, small amounts of LF, EF, and other anthrax components are present in varying amounts in the final product and are responsible for a variety of undesirable medical side-effects [11].

As PA alone is responsible for the protective effects of the anthrax vaccine, there has been considerable research into the production of recombinant PA (rPA) for a more defined, improved vaccine. Expression of rPA in *Escherichia coli* has been associated with a number of technical difficulties. Initial approaches yielded a very low amount of expressed protein [12], while improved conditions provided larger amounts of rPA in the form of insoluble, inactive inclusion bodies [13]. While these issues appear to have been resolved [14], recombinant protein from *E. coli* is contaminated with trace amounts of endotoxin, which provides another processing problem before human use is possible. Baculovirus or vaccinia virus expression systems yield only small amounts of soluble rPA and are expensive to scale-

up [15]. *Bacillus subtilis* has been found to be an effective host for the expression of soluble, active rPA without the presence of endotoxin [16-18]. Moreover, the anthrax PA signal sequence is recognised by *B. subtilis* and rPA is actively excreted into the culture medium. Unfortunately, *B. subtilis* encodes numerous proteases that degrade expressed recombinant proteins, including rPA [19,20]. Therefore, expression in *B. subtilis* requires the deletion of several proteases or the purification of rPA in the presence of protease inhibitors that are not compatible with human use [18,21].

In this report, we describe the successful expression of rPA in *Bacillus megaterium* and the purification of the recombinant protein from culture supernatants. As an expression system *B. megaterium* has the advantages of non-pathogenicity, a lack of endotoxin, no major protease activity, and a commercial, inducible expression vector based on the xylose promoter [22]. Therefore, proteins produced from *B. megaterium* do not need to be purified in the presence of toxic protease inhibitors and should be suitable for human use with a minimum of downstream processing.

Materials and Methods

Cells and Reagents

Escherichia coli XL10 was obtained from Stratagene (La Jolla, CA, USA) and *B. megaterium* WH320 and plasmid pWH1520 were acquired from MoBiTec (Marcos Island, FL, USA). Both cell types were routinely cultured in Luria-Bertani broth or on Luria-Bertani plates, with 50 µg/ml carbenicillin or 10 µg/ml tetracycline when required. Total DNA was isolated by the phenol chloroform method from *B. anthracis* Ames grown to stationary phase in Nutrient Broth, and was resuspended in distilled water [23]. All chemicals were obtained from Sigma-Aldrich (Oakville, ON, CA) unless otherwise stated.

Construction of pWH1520b

In order improve the general utility of pWH1520 as an expression vector, an affinity tag and extra restriction endonuclease sites were added near the 5'-end of the *xylA'* gene. The decahistidine tag, enterokinase site and multiple cloning site from pET19m [24] were amplified by PCR utilising 5'-ATCGATACTAGTGGCCATCATCATCATCATCATCA as the forward primer (with the SpeI site underlined) and 5'-ATCGATGGATCCCCACGTGC CCGGGCGTTTAAA as the reverse primer (with the BamHI site underlined). The PCR reaction was performed using a 5:1 mixture of Taq (Fisher Scientific; Nepean, ON, CA) and Pfu (Fermentas; Burlington, ON, CA) polymerases and an initial denaturation of 1.5 min at 95°C, 30 cycles of 95°C /55°C/72°C for one minute each, and a final extension of 10 min at 72°C. The 182 bp product was purified from a 1.4% agarose gel using the QiaQuick kit (Qiagen; Mississauga, ON, CA), and was digested with SpeI and BamHI (Promega; Madison, WI, USA). The digested insert was ligated into SpeI/BamHI digested, alkaline phosphatase (Promega) treated pWH1520 using the Fermentas Rapid DNA ligation kit. The resulting plasmid, pWH5120b (Figure 1), was transformed into *E. coli* XL10 cells. The sequence of the plasmid was confirmed by PCR cycle sequencing as outlined in the manufacturer's instructions (Applied Biosystems; Mississauga, ON, CA).

Cloning of *B. anthracis* Protective Antigen

PA from *B. anthracis* Ames was amplified by PCR using the conditions described above and 5'-GGATCATGCGGCCGCGAATGAAAAAACGAAAAGTGTTAATA as the forward primer (with the NotI site underlined) and 5'-ATCGATGGATCCTTATCCTATCTATA GCCTTTTTTA as the reverse primer (with the BamHI site underlined). The 2341 bp product was purified from a 1% agarose gel and digested with NotI and BamHI (Promega). The insert was ligated into NotI/BamHI digested, alkaline phosphatase treated pWH1520b and transformed into *E. coli* XL10 cells. The plasmid was recovered from these cells using the Qiaprep SpinMini kit (Qiagen) and the DNA sequence confirmed by PCR cycle sequencing.

The recombinant plasmid was transformed into *B. megaterium* protoplasts using the methods of Puyet et al. [25]. Briefly, *B. megaterium* WH320 was grown in AB3 medium

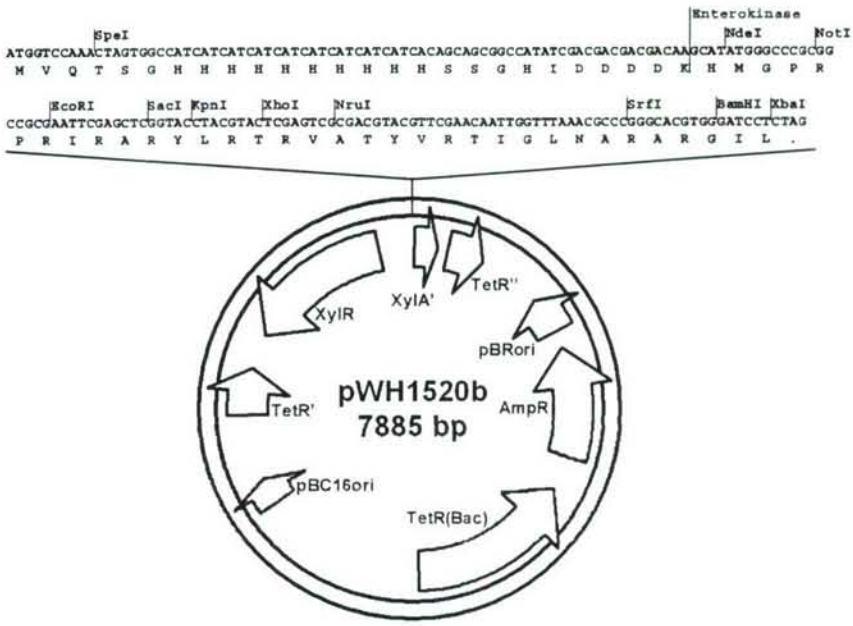

Figure 1. *Vector Diagram of pWH1520b. The vector was derived from pWH1520 [22] via the addition of a polyhistidine tag, enterokinase cleavage site, and additional restriction endonuclease sites from pET19m [24]. The 5' end of the xylA' gene, which contains the tag and multiple cloning site is shown in detail. Inactivation of the XylR repressor protein by the addition of xylose leads to the expression of the XylA' fusion protein.*

(Becton Dickinson; Sparks, MD, USA) at 37°C and 250 rpm until A_{600nm} reached 1.0. The cells were centrifuged and resuspended in 5.0 ml of SMMP (AB3 medium containing 500 mM sucrose, 20 mM sodium maleic acid, 5 mM $MgCl_2$) containing 2 mg/ml lysozyme (EM Science; Gibbstown, NJ, USA). This suspension was incubated for 90 min at 37°C and 100 rpm and then centrifuged at 2000 x g for 10 min at 20°C. The pelleted protoplasts were resuspended in 5.0 ml SMMP and stored at -70°C in 0.5 ml aliquots. When required, an aliquot of protoplasts was thawed on ice and mixed with 5 µl of recombinant plasmid before the addition of 1.5 ml of 40% polyethylene glycol 8000 in SMM (500 mM sucrose, 20 mM sodium maleic acid, 5 mM $MgCl_2$, pH 6.5). The sample was then incubated for 2 min at room temperature before the addition of 5.0 ml SMMP and centrifugation at 1550 x g for 10 min at 20°C. The pellet protoplasts were resuspended in 0.5 ml SMMP and incubated at 37°C and 100 rpm for 90 min to allow expression of tetracycline resistance. 200 µl aliquots were then gently spread on CR5 plates (103 g/L sucrose, 0.25 g/L K_2SO_4, 10 g/L $MgCl_2$-$6H_2O$, 10 g/L glucose, 6.5 g/L 4-morpholinepropanesulfonic acid, 0.66 g/L NaOH, 6 g/L proline, 0.2 g/L casamino acids, 10 g/L yeast extract, 0.05 g/L KH_2PO_4, 2.2 g/L $CaCl_2$, 80 µg/ml $ZnCl_2$, 400 µg/ml $FeCl_3$-$6H_2O$, 20 µg/ml $CuCl_2$-$2H_2O$, 20 µg/ml $MnCl_2$-$4H_2O$, 20 µg/ml $(NH_4)_6Mo_7O_{24}$-$4H_2O$, and 1.1% w/v agar) containing tetracycline before incubation overnight at 37°C. The resulting colonies were picked into Luria-Bertani broth containing tetracycline and incubated at 37°C and 250 rpm. Recombinant plasmids were isolated and confirmed using the Qiaprep Spin Mini kit with the addition of an initial 1 hr incubation at 37°C in buffer P1 containing 10 mg/ml lysozyme.

Experiments were also performed using transformation by electroporation as outlined in Moro et al. [26]. *B. megaterium* WH320 cells were grown in 100 mL of LB broth until the $A_{600\ nm}$ reached 1.0, after which the culture was centrifuged at 2000 x g for 20 minutes. The pelleted cells were washed once in electroporation buffer (EB), consisting of 25% PEG 8000 and 100 mM sorbitol, and resuspended in the same EB. For electroporation, a 0.1 cm gapped cuvette was used in a Bio-Rad Micro-pulse electroporation apparatus (Mississauga, ON, CA) with a constant resistance of 10 μF. Different samples were electroporated at 0.75, 1.00, 1.25 and 1.50 kV for 5.0 milliseconds. 1.0 ml of LB broth was added to the electroporated culture before incubation at 37°C and 250 rpm. 100 μl aliquots were plated on LB agar containing tetracycline and incubated overnight at 37°C. Isolated colonies were grown out overnight at 37°C and 250 rpm in LB broth containing tetracycline and the plasmid isolated as described above.

Expression and Purification of Recombinant Protective Antigen

Transgenic *B. megaterium* was grown in LB broth containing tetracycline at 37°C and 250 rpm until the A_{600nm} reached 0.3. Xylose was then added to a final concentration of 0.5% w/v and the incubation continued at 37°C and 250 rpm for an additional 3 hr. The samples were then centrifuged at 3500 x g for 20 min at 4°C and the pelleted cells and spent medium separated. The cells were resuspended in a minimal amount of distilled water, frozen at -20°C, thawed, and sonicated on ice with a Branson 450 probe sonicator (Dander, CA, USA). The homogenised cells were then centrifuged at 3500 x g for 20 min at 4°C to separate soluble and insoluble components. Aliquots of the cell fractions and spent culture medium were then analysed by SDS polyacrylamide gel electrophoresis as described below.

The spent medium from larger (500 ml) induced cultures was concentrated by 50 psi argon pressure and an Amicon model 402 cell (Lexington, MA, USA) equipped with a 30 kDa molecular weight cut-off polysulfone filter (Sartorius; Gottingen, Germany). This concentrate was then dialysed against 20 mM Tris-HCl pH 7.8 at 4°C. The sample was loaded onto a 1.6 x 10 cm Q-Sepharose FF column (Amersham; Baie d'Urfe, QB, CA) connected to a Pharmacia FPLC (Amersham) and equilibrated with 20 mM Tris-HCl pH 7.8. The column was eluted using a step gradient of 20 mM Tris-HCl pH 7.8 containing 100, 200, 300, 400, 500, 600, or 1000 mM KCl. Column effluent was monitored at A_{280nm} and samples of each fraction were analysed by SDS polyacrylamide gel electrophoresis as described below. Fractions containing purified rPA were pooled, concentrated using a 50 kDa molecular weight cut-off centripetal filter (Pall; Ann Arbor, MI, USA), and stored at -20°C with or without 20% v/v glycerol. Protein concentration was determined using the Bio-Rad dye.

All gel electrophoresis was performed using a Mini-Protean II cell (Bio-Rad; Mississauga, ON), and a 7.5% acrylamide resolving gel with a 5% stacking gel. Samples were denatured and reduced using SDS and dithiothreitol. All gels were stained with Coomassie Brilliant Blue R250. Electroblotting of purified rPA was performed on an unstained SDS polyacrylamide gel with Sequiblot PVDF (Bio-Rad) membranes and a transfer buffer of 10 mM Tris, 100 mM glycine, and 10% methanol. The sample was transferred at 100V for 1 hr, the PVDF washed 10 times in distilled water, and the membrane stained with Amido Black. The rPA band was excised, dried under vacuum, and stored at 4°C.

N-terminal amino acid sequencing was performed by the pulsed-liquid method using an ABI model 470 sequencer (Applied Biosystems; Foster City, CA, USA) according to the manufacturer's instructions. The electroblotted rPA band was sequenced directly, while an aliquot of the concentrated rPA solution was transferred to PVDF using a Pro-Sorb cartridge (Applied Biosystems) as per the manufacturer's instructions. N-terminal deblocking by pyroglutamate aminopeptidase was also performed on a duplicate electroblotted rPA band prior to N-terminal sequencing. The band was incubated with 200 µl 0.5% w/v polyvinylpyrrolidine-360, 0.1% acetic acid for 20 min at room temperature, washed 3 times with distilled water, and incubated with 40 µl 50 mM phosphate buffer pH 7.0, 10 mM DTT, 1 mM EDTA, 1 U *Pyrococcus furiosus* pyroglutamate aminopeptidase (TaKaRa; Madison, WI, USA) at 90°C for 1 hr. The band was then washed 3 times with distilled water, dried under vacuum, and stored at 4°C.

Results and Discussion

After successful cloning of the complete *B. anthracis* Ames PA gene into pWH1520b, the recombinant plasmid was transformed into *B. megaterium* 320. Initial attempts at introducing the plasmid via electroporation, as outlined in Moro et al. [26], were unsuccessful regardless of the voltage conditions or electroporation medium. With the exception of Moro et al., there have been no literature reports of successful electroporation of *B. megaterium*. Until the Moro et al. procedure has been reproduced in other laboratories, *B. megaterium* should continue to be considered refractory to electroporation. In contrast, all chemical transformations of *B. megaterium* protoplasts, as per the method of Puyet et al. [25], were successful with a high efficiency.

Small, 5.0 ml pilot-scale inductions of transgenic *B. megaterium* were performed by the addition of xylose to a final concentration of 0.5% w/v after the bacteria had grown to a density of 0.3 A_{650nm}. Examination of soluble and insoluble cell fractions failed to show any detectable induction of rPA within the bacterial cells (Figure 2A). However, a comparable examination of culture medium clearly showed the presence of an approximately 83 kDa band in the induced cultures only (Figure 2B). This extracellular localisation is consistent with the expression of native and recombinant PA in *B. anthracis* [27], and with the expression of rPA in *B. subtilis* [18]. Therefore, *B. megaterium* recognizes the sec-dependent signal sequence found on full-length rPA [28].

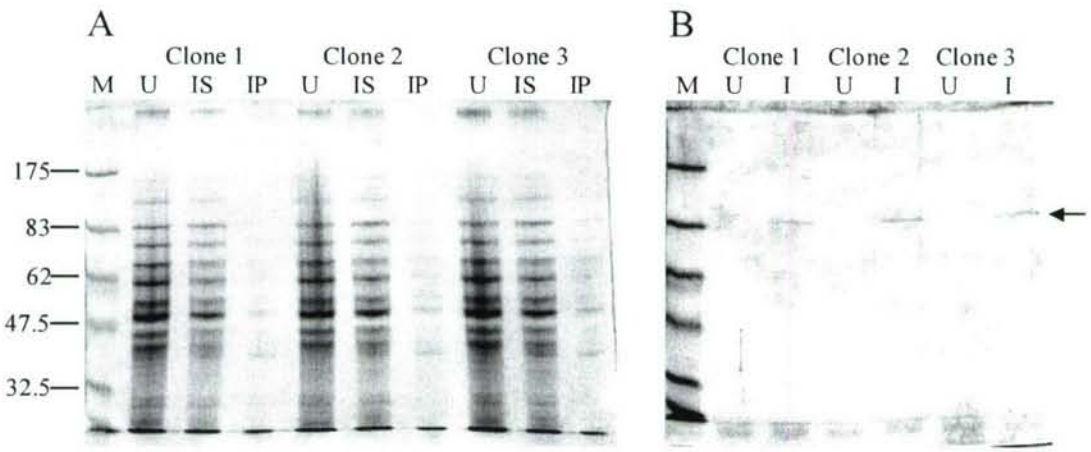

Figure 2. *The production of recombinant protective antigen by Bacillus megaterium. 5.0 ml cultures of B. megaterium containing pWH1520b-rPA were grown in the presence or absence of 0.5% xylose as outlined in the Material and Methods section. (A) Polyacrylamide gel electrophoresis of the cellular material from three independent clones, and (B) electrophoresis of the corresponding culture supernatants. (U) Represents uninduced cell material or culture supernatant, (IS) induced, soluble cell material, (IP) induced, insoluble cell material, (I) induced culture supernatant, and (M) mass standards.*

The induction of rPA was scaled up to 500 ml with all other conditions kept constant, and the resulting spent culture medium concentrated down to approximately 3 ml using a 30 kDa cut-off filter (Figure 3). The concentrated medium was found to contain three lower molecular weight contaminants of approximately 50, 48, and 38 kDa. These contaminating bands might represent degradation products of the rPA, or might be other proteins excreted during *B. megaterium* growth. The contaminant pattern does not, however, resemble the degradation pattern seen in culture supernatants from *B. subtilis* expressing rPA [18]. In order to remove the contaminants, the concentrated rPA was dialysed against 20 mM Tris-HCl pH 7.8 and subjected to ion-exchange chromatography (Figure 3). Electrophoretically pure rPA was eluted from the column with 100 mM NaCl. This material was assayed for protein concentration, and 480 µg of rPA was found to have been produced in the 500 ml of culture medium after only 2.5 hr of induction. The yield of rPA should be easily increased by altering several induction conditions.

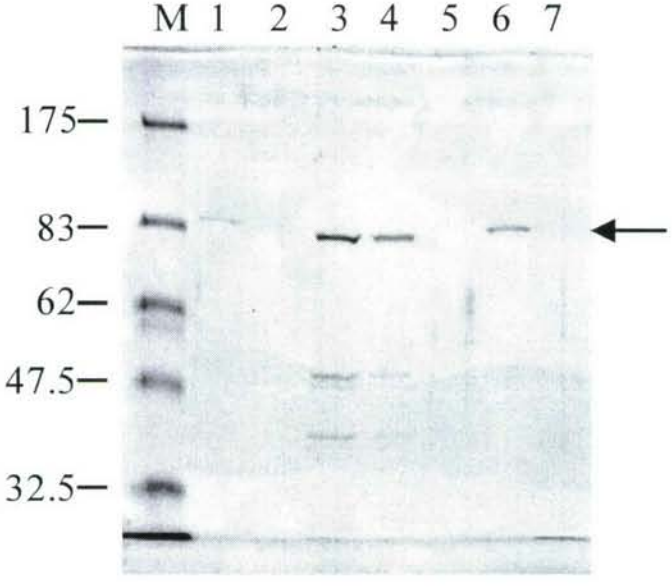

Figure 3. *Purification of recombinant protective antigen. A 500 ml culture of B. megaterium carrying pWH1520b-rPA was grown and induced with 0.5% xylose as described in the Materials and Methods section. Polyacrylamide gel electrophoresis was performed on various fractions during purification of rPA. (M) Protein mass standards, (1) unconcentrated culture supernatant, (2) <30 kDa fraction, (3) >30 kDa fraction, (4) >30 kDa fraction after dialysis against 20 mM Tris-HCl pH 7.8, (5) Q-Sepharose eluate after loading the dialysed >30 kDa fraction, (6) Q-Sepharose eluate after washing with 20 mM Tris-HCl/100 mM NaCl, and (7) Q-Sepharose eluate after washing with 20 mM Tris-HCl/200 mM NaCl.*

The purified rPA was subjected to amino acid sequencing and yielded the following as an N-terminal sequence: EVKQENRLLNESES. This sequence is identical to the predicted N-terminus for the mature PA [18]. No secondary sequence was detected, demonstrating that the purified material contained only rPA and that the protein did not suffer from the 5 amino acid truncation seen in rPA produced by *B. subtilis* WB600 in the absence of protease inhibitors [18]. The purified rPA was found to be equivalent to purified native PA in an ELISA assay system (D. Fornika and L. Nagata, personal communication), suggesting that the rPA should be effective as a vaccine antigen.

The results of the present experiments have yielded several interesting conclusions and point the way for future studies. The most important conclusion from the standpoint of this laboratory is the fact that, as a heterologous expression system, *B. megaterium* can produce soluble, full-length protein which is known to be insoluble in *E. coli* expression systems and proteolytically degraded in *B. subtilis* systems. This feature should be of great assistance in our on-going studies with other difficult recombinant proteins from a variety of sources. Secondly, it is clear that *B. megaterium* recognizes the sec-dependent signal sequence on the *B. anthracis* full-length PA and excretes the mature protein into the culture medium. It is not clear whether *B. megaterium* recognizes all *Bacillus* sec-dependent prosequences, but the heterogeneity of known sec-dependent signal sequences [28] would suggest that most, if not all, should function in *B. megaterium*. The functionality of the PA prosequence is currently being exploited in our laboratory to construct a further derivative of pWH1520b which contains the export sequence upstream of the polyhistidine tag. This vector should then function as a general purpose *B. megaterium* extracellular expression system for any recombinant protein.

In the specific case of rPA expression by *B. megaterium*, several future experiments are important. The length of time of induction should be examined from 2 hr to several days in order to determine the optimal time frame for maximal yield of intact rPA. The amount of tetracycline, xylose and media used for induction should also be varied. In particular, the use of a fully defined, minimal, animal product-free medium should be tested, as successful induction with such a medium would be highly desirable from a production standpoint. The stability of the rPA in the culture supernatant should be closely examined, and, finally, the purification process could be simplified. These important experiments would be an ideal project for a Defence Research Associate or co-op student.

References

1. Borio, L., Frank, D., Mani, V., Chiriboga, C., Pollanen, M., Ripple, M., Ali, S., DiAngelo, C., Lee, J., Arden, J., Titus, J., Fowler, D., O'Toole, T., Masur, H., Bartlett, J., and Inglesby, T. (2001). Death due to bioterrorism-related inhalational anthrax: report of 2 patients. *JAMA*, 286, 2554-2559.

2. Mayer, T. A., Bersoff-Matcha, S., Murphy, C., Earls, J., Harper, S., Pauze, D., Nguyen, M., Rosenthal, J., Cerva, D. Jr, Druckenbrod, G., Hanfling, D., Fatteh, N., Napoli, A., Nayyar, A., and Berman, E. L. (2001). Clinical presentation of inhalational anthrax following bioterrorism exposure: report of 2 surviving patients. *JAMA*, 286, 2549-2553.

3. Makino, S., Uchida, I., Terakado, N., Sasakawa, C., and Yoshikawa, M. (1989). Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in *Bacillus anthracis*. *Journal of Bacteriology*, 171, 722-730.

4. Pannucci, J., Okinaka, R. T., Williams, E., Sabin, R., Ticknor, L. O., and Kuske, C. R. (2002). DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria. *BMC Genomics*, 3, 34.

5. Okinaka, R. T., Cloud, K., Hampton, O., Hoffmaster, A. R., Hill, K. K., Keim, P., Koehler, T. M., Lamke, G., Kumano, S., Mahillon, J., Manter, D., Martinez, Y., Ricke, D., Svensson, R., and Jackson, P. J. (1999). Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. *Journal of Bacteriology*, 181, 6509-6515.

6. Duesbery, N. S., Webb, C. P., Leppla, S. H., Gordon, V. M., Klimpel, K. R., Copeland, T. D., Ahn, N. G., Oskarsson, M. K., Fukasawa, K., Paull, K. D., and Vande Woude, G. F. (1998). Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science*, 280, 734-737.

7. O'Brien, J., Friedlander, A., Dreier, T., Ezzell, J., and Leppla, S. (1985). Effects of anthrax toxin components on human neutrophils. *Infection and Immunity*, 47, 306-310.

8. Sellman, B. R., Mourez, M., and Collier, R. J. (2001). Dominant-negative mutants of a toxin subunit: an approach to therapy of anthrax. *Science*, 292, 695-697.

9. Puziss, M. and Wright, G. G. (1963). Studies on immunity in anthrax. X. Gel-adsorbed protective antigen for immunization of man. *Journal of Bacteriology*, 85, 230-236.

10. Puziss, M. and Wright, G. G. (1954). Studies on immunity in anthrax. IV. Factors influencing elaboration of the protective antigen of *Bacillus anthracis* in chemically defined media. *Journal of Bacteriology*, 68, 475-482.

11. Jendrek, S., Little, S. F., Hem, S., Mitra, G., and Giardina, S. (2003). Evaluation of the compatibility of a second generation recombinant anthrax vaccine with aluminum-containing adjuvants. *Vaccine*, 21, 3011-3018.

12. Singh, Y., Chaudhary, V. K., and Leppla, S. H. (1989). A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action in vivo. *Journal of Biological Chemistry,* 264, 19103-19107.

13. Gupta, P., Waheed, S. M., and Bhatnagar, R. (1999). Expression and purification of the recombinant protective antigen of *Bacillus anthracis*. *Protein Expression and Purification,* 16, 369-376.

14. Ahuja, N., Kumar, P., and Bhatnagar, R. (2001). Rapid purification of recombinant anthrax-protective antigen under nondenaturing conditions. *Biochemical and Biophysical Research Communications,* 286, 6-11.

15. Iacono-Connors, L. C., Schmaljohn, C. S., and Dalrymple, J. M. (1990). Expression of the *Bacillus anthracis* protective antigen gene by baculovirus and vaccinia virus recombinants. *Infection and Immunity,* 58, 366-372.

16. Ivins, B. E. and Welkos, S. L. (1986). Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. *Infection and Immunity,* 54, 537-542.

17. Baillie, L., Moir, A., and Manchee, R. (1998). The expression of the protective antigen of *Bacillus anthracis* in *Bacillus subtilis*. *Journal of Applied Microbiology,* 84, 741-746.

18. Cohen, S., Mendelson, I., Altboum, Z., Kobiler, D., Elhanany, E., Bino, T., Leitner, M., Inbar, I., Rosenberg, H., Gozes, Y., Barak, R., Fisher, M., Kronman, C., Velan, B., and Shafferman, A. (2000). Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax. *Infection and Immunity,* 68, 4549-4558.

19. Baillie, L. W., Johnson, M., and Manchee, R. J. (1994). Evaluation of *Bacillus subtilis* strain IS53 for the production of *Bacillus anthracis* protective antigen. *Letters in Applied Microbiology,* 19, 225-227.

20. Thwaite, J. E., Baillie, L. W., Carter, N. M., Stephenson, K., Rees, M., Harwood, C. R., and Emmerson, P. T. (2002). Optimization of the cell wall microenvironment allows increased production of recombinant *Bacillus anthracis* protective antigen from *B. subtilis*. *Applied and Environmental Microbiology,* 68, 227-234.

21. Wu, X. C., Lee, W., Tran, L., and Wong, S. L. (1991). Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *Journal of Bacteriology,* 173, 4952-4958.

22. Rygus, T. and Hillen, W. (1991). Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. *Applied Microbiology and Biotechnology,* 35, 594-599.

23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1987). Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Press, Plainview, NY, USA.

24. Venos, E. S. , Knodel, M. H., Radford, C. L., and Berger, B. J. (2003). Characterisation of potential antimicrobial targets for tuberculosis. II. Branched-chain amino acid aminotransferase and methionine regeneration in *Mycobacterium tuberculosis*. (DRDC Suffield TR2003-147). Defence R&D Canada – Suffield.

25. Puyet, A., Sandoval, H., Lopez, P., Aguilar, A., Martin, J. F., and Espinosa, M. (1987). A simple medium for rapid regeneration of *Bacillus subtilis* protoplasts transformed with plasmid DNA. *FEMS Microbiol Letts,* 40, 1-5.

26. Moro, A., Sanchez, J. C., and Serguera, C. (1995). Transformation of *Bacillus megaterium* by electroporation. *Biotechnol Tech,* 9, 589-590.

27. Ramirez, D. M., Leppla, S. H., Schneerson, R., and Shiloach, J. (2002). Production, recovery and immunogenicity of the protective antigen from a recombinant strain of *Bacillus anthracis*. *Journal of Industrial Microbiology and Biotechnology,* 28, 232-238.

28. Tjalsma, H., Bolhuis, A., Jongbloed, J. D., Bron, S., and van Dijl, J. M. (2000). Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiology and Molecular Biology Reviews,* 64, 515-547.

List of symbols/abbreviations/acronyms/initialisms

DND	Department of National Defence
PA	*Bacillus anthracis* protective antigen
rPA	Recombinant *Bacillus anthracis* protective antigen

DOCUMENT CONTROL DATA

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (the name and address of the organization preparing the document. Organizations for who the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in Section 8.)

   Defence R&D Canada – Suffield

2. SECURITY CLASSIFICATION (overall security classification of the document, including special warning terms if applicable)

   UNCLASSIFIED

3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S, C or U) in parentheses after the title).

   Expression of *Bacillus anthracis* protective antigen in *Bacillus megaterium* (U)

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

   Berger, Bradley J., Schwandt, Kerrie E., and Radford, Cynthia L.

5. DATE OF PUBLICATION (month and year of publication of document)

   March 2004

6a. NO. OF PAGES (total containing information, include Annexes, Appendices, etc) 25

6b. NO. OF REFS (total cited in document) 28

7. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

   Technical Memorandum

8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

   Defence R&D Canada – Suffield

9a. PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant.)

   CBD-01-013

9b. CONTRACT NO. (If appropriate, the applicable number under which the document was written.)

10a. ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.)

   DRDC Suffield TM 2004-045

10b. OTHER DOCUMENT NOs. (Any other numbers which may be assigned this document either by the originator or by the sponsor.)

11. DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification)

   (x) Unlimited distribution
   ( ) Distribution limited to defence departments and defence contractors; further distribution only as approved
   ( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved
   ( ) Distribution limited to government departments and agencies; further distribution only as approved
   ( ) Distribution limited to defence departments; further distribution only as approved
   ( ) Other (please specify):

12. DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally corresponded to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected).

13. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C) or (U). It is not necessary to include here abstracts in both official languages unless the text is bilingual).

Bacillus anthracis protective antigen is the central component of the anthrax toxin complexes that facilitates entry of lethal factor and edema factor into host cells. Protective antigen is also the major immunogenic component present in the currently licensed anthrax vaccine. In order to produce full-length, soluble protective antigen, the gene has been cloned and expressed using *Bacillus megaterium* and a xylose-inducible heterologous expression system. After only 3.5 hours growth post-induction in Luria-Bertani broth, the transgenic *B. megaterium* were found to secrete approximately 1 μg/ml protective antigen into the culture medium. The recombinant protein was easily purified to homogeneity in a single step by ion exchange chromatography. N-terminal amino acid sequencing of the final product confirmed that the recombinant protective antigen was full-length and that no proteolytic degradation had occurred.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifies, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

Bacillus anthracis, *Bacillus megaterium*, protective antigen, recombinant protein, purification, amino acid sequencing

Defence R&D Canada

Canada's leader in defence
and national security R&D

R & D pour la défense Canada

Chef de file au Canada en R & D
pour la défense et la sécurité nationale

DEFENCE R&D DÉFENSE

www.drdc-rddc.gc.ca